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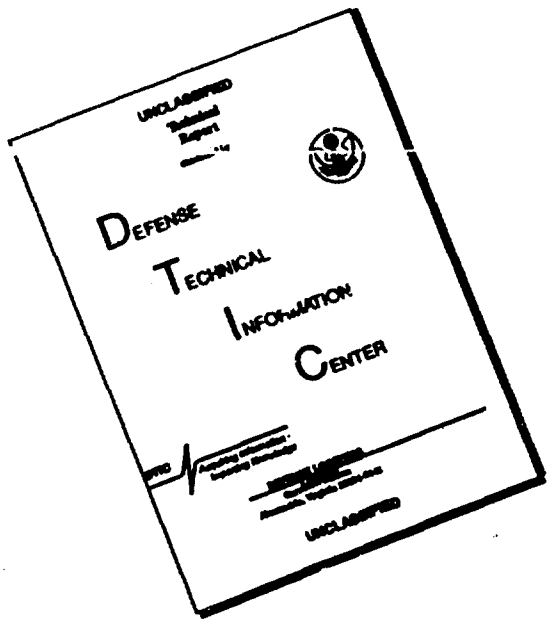

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INFLUENCE OF ACTINOMYCIN D ON THE RNA METABOLISM OF ANTIBODY-FORMING SPLEEN CELLS IN VITRO*

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Hermann Moreau**
Hans Krüger

1. Uridine-2-^{14}C *in vitro* is relatively rapidly incorporated into the RNA of spleen cells of rabbits. The highest amount of radioactivity is found in the 65° C fraction. Antibody-forming cells are especially active in RNA synthesis.

2. Actinomycin D strongly inhibits the RNA synthesis. While cells of normal rabbits are affected particularly in the 65° C fraction, in immunized animals the incorporation of uridine-2-^{14}C is inhibited in all fractions.

In an earlier work we were able to show that actinomycin D inhibits the *in vitro* synthesis of antibodies against alcohol dehydrogenase [1,2]. This finding suggested that a DNA-dependent RNA synthesis is necessary for the formation of antibodies. [See the list of abbreviations on page 2.] In the present article it is shown that actinomycin D inhibits RNA synthesis in the spleen cells. The experiments were carried out with cells from rabbits that had received an antigen injection before killing and 10-12 weeks after immunization with alcohol dehydrogenase.

Methods

Immunization with ADH, isolation of the spleen cells from rabbits, and determination of the antibody were carried out in accordance with [2,3].

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Short-Term Markings

6-7 g of spleen cells were pre-incubated in 120 ml of Eagle's medium for one hour at 37° C with and without actinomycin D (5 µg/ml), and then incubated for 20 minutes with uridine 2-^{14}C (1 µC/6 ml). An equal volume of ice-cold NaCl solution (0.14 M) was then added and the mixture centrifuged (10 minutes at 10,000 x g).

Isolation of RNA

The spleen cells obtained were homogenized in the Potter-Elvehjem apparatus with ten times their volume of ice-cold NaCl solution (0.14 M) with the addition of 1 mg/ml of bentonite (pretreated as in [4]). The mixture was then centrifuged (15 minutes at 800 x g), the cytoplasmatic liquid decanted, and the sediment washed twice more with NaCl solution.

Recovery of the Cytoplasmatic RNA. -- To the combined liquid was added up to 0.5% sodium desoxycholate and an equal volume of phenol (80%; pH 6.0). After 20 minutes' agitation at 4° C and centrifuging (10 minutes at 10,000 x g) the watery phase was pipetted off and the interphase again treated with ten times its volume of NaCl solution and a corresponding quantity of phenol.

The watery phases were mixed with alcohol up to 67%. This solution was kept overnight in the cold storage room. The RNA centrifuged off was dissolved in tris-HCl buffer (0.01 M; pH 7.4) with 0.001 M $MgCl_2$, precipitated three times by means of potassium acetate (final concentration: 2 M) and ethanol (final concentration: 20%) and washed twice more with ethanol (95%).

The purified RNA was then dissolved in tris-HCl buffer (0.01 M; pH 7.4) with 0.001 M $MgCl_2$.

Recovery of Nuclear RNA. -- The nuclear sediment was homogenized with ten times its volume of NaCl solution (0.14 M) and then added to an equal volume of phenol (80%; pH 6.0). RNA fractions were then obtained from this by the method of Georgiev et al. [5] at 4°, 50°, and 65° C. The corresponding RNA's were isolated from the watery phases in each case as described above for the recovery of the cytoplasmatic RNA.

The radioactivity was measured with the methane flow-

Abbreviations:

ADH - alcohol dehydrogenase
DNA - deoxyribonucleic acid
RNA - ribonucleic acid
Tris - tris(hydroxymethyl)-aminomethane
ipm - impulses per minute
OD - optical density
rpm - revolutions per minute

through counter tube. The specific radioactivity is given as ipm/OD (measured at 260 mμ; thickness of layer 10 mm).

Gradient Centrifuging

This was done in accordance with the methods of Britten et al. [6]. 8 OD of the isolated RNA, dissolved in 0.5 ml of tris-HCl buffer (0.01 M; pH 7.4) with 0.001 M $MgCl_2$, was set to a saccharose gradient (4.5 ml; 5-20% saccharose in 0.01 M sodium acetate, pH 5.1; 0.1 M NaCl; 0.001 M $MgCl_2$) and centrifuged in the preparation ultracentrifuge (Spinco L 50) in the SW 39 rotor for five hours at 39,000 rpm. The centrifuge tube was tapped and the contents caught by drops in separate tubes.

The content of the individual tubes was diluted with 1 ml of H_2O, the extinction obtained at 260 mμ, and an aliquot transferred to aluminum dishes. The radioactivity was determined with the methane flow-through counter tube.

Other Methods

The base ratio was analysed by the method of Markham et al. [7]. We thank Dr.Dr. U. Hagen, of the Radiological Institute of the University of Freiburg im Breisgau, for determining the sedimentation constants.

Preparations

We obtained ADH from C.F. Boehringer & Soehne GmbH, Mannheim; Eagle's medium from Grand Island Biological Co., Grand Island, N.Y.; uridine-2-^{14}C (30 mC/mMol), uracil-2-^{14}C (20 mC/mMol), and orotic acid 6-^{14}C (8 mC/mMol) from New England Nuclear Corporation, Boston, Massachusetts. We have the firm of Merck, Sharp & Dohme, Rahway, N.J., to thank for the actinomycin D.

Results

1. Influence of actinomycin D on antibody synthesis. -- Isolated spleen cells incubated in Eagle's medium form antibodies against alcohol dehydrogenase which can be shown up by the enzymatic optical method [2]. This synthesis is inhibited by actinomycin D even in a concentration of 5 μg/ml (cf. Table 1).

Table 1

Synthesis of Antibodies Against ADH in Vitro Under the Influence of Actinomycin D. The cells from immunized animals were incubated in Eagle's medium for 20 hours. (For details see [2].)

Addition of	Inhibition of the ADH Activity as Compared to Control Animals
--	70%
5 μg/ml of actinomycin D	0%

Table 2

Influence of Actinomycin D (5 µg/ml) on the incorporation of Uridine-2-^{14}C into RNA from Spleen Cells from Control Animals and Immunized Animals (in i.u./CE)

	Cytoplasmatic Fraction		4°C Fraction		50°C Fraction		65°C Fraction	
		+Actinomycin D		+Actinomycin D		+Actinomycin D		+Actinomycin D
Control Animals	120	120	186	147	545	340	840	412
Immunized Animals	320	140	210	198	920	418	1430	630

2. Influence of actinomycin D on the RNA synthesis. -- While the isolated cells hardly incorporate uracil-2-^{14}C and orotic acid-6-^{14}C into the RNA at all, uridine-2-^{14}C is very rapidly taken up. For that reason uridine-2-^{14}C was used for the short-term markings.

After 20 minutes' incubation with uridine-2-^{14}C all the RNA fractions obtained by Georgiev's method [5] are marked. From Table 2 it may be seen that the RNA fractions from cells of immunized animals show a substantially higher specific activity than those from control animals. In both cases the highest specific activity is found in the 65° C fraction. This RNA approaches DNA in its base ratio. The greater part shows a Svedberg constant of $S_{20} = 8$.

Actinomycin D has a definite influence on the synthesis of the RNA isolated at 65° C. The incorporation of uridine-2-^{14}C is reduced by more than half. (Cf. Table 2.) This result is also clear from Figures 1 and 2, where the sedimentation behavior of this RNA in a saccharose gradient (5-20%) is shown.

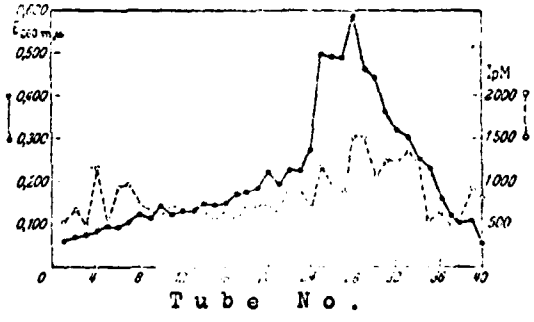

Figure 1. Sedimentation diagram of RNA (65° C fraction) from spleen cells.

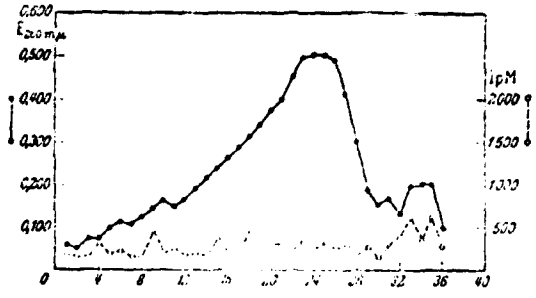

Figure 2. Sedimentation diagram of RNA (65° C fraction) from spleen cells under the influence of actinomycin D (5 μg/ml).

But actinomycin D also shows a definite effect on the incorporation of uridine-2-^{14}C into the other RNA fractions (cf. Table 2). The effect on the fractions from immunized animals is substantially greater than on the controls.

Discussion

The inhibition of antibody synthesis *in vitro* by actinomycin D is accompanied by a definite reduction in the RNA synthesis. Both the nuclear RNA and the cytoplasmatic RNA are affected by this inhibition of synthesis. This finding is quite in harmony with the latest investigations of Smiley et al. [8].

From the above findings it ~~may be~~ concluded that in spleen cells from immunized animals the synthesis of antibodies is preceded by the formation of a DNA-dependent RNA.

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